An enantiomer-based virtual screening approach: Discovery of chiral organophosphates as acetyl cholinesterase inhibitors

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ABSTRACT

Chiral organophosphates (OPs) have been used widely around the world, very little is known about binding mechanisms with biological macromolecules. An in-depth understanding of the stereo selectivity of human AChE and discovering bioactive enantiomers of OPs can decrease health risks of these chiral chemicals. In the present study, a flexible molecular docking approach was conducted to investigate different binding modes of twelve phosphorus enantiomers. A pharmacophore model was then developed on basis of the bioactive conformations of these compounds. After virtual screening, twenty-four potential bioactive compounds were found, of which three compounds (Ethyl *p*-nitrophenyl phenylphosphonate (EPN), 1-naphthaleneacetic anhydride and *N*,4-dimethyl-*N*-phenyl-benzenesulfonamide) were tested by use of different *in vitro* assays. *S*-isomer of EPN was also found to exhibit greater inhibitory activity towards human AChE than the corresponding *R*-isomer. These findings affirm that stereochemistry plays a crucial role in virtual screening, and provide a new insight into designing safer organ phosphorus pesticides on human health.

1. Introduction

Organophosphates (OPs) have been used to treat various neurological diseases such as Alzheimer's disease, as well as being used to prepare insecticides and fire retardant materials (Brown et al., 1989). Given that OPs are used in such a wide range of applications, questions continue to be asked regarding their biological effects, duration of action and toxic mechanisms (Liu et al., 2005). Modern OPs-based pesticides that contained one or more chiral atoms were reported to show different toxic potency to insects or human (Tenberken et al., 2010; Tsai et al., 2010; Wang et al., 2010; Zhang et al., 2014). Currently, the OPs stockpile worldwide is estimated at 200,000 t (Singh, 2009). These concerns about health and the environment have encouraged the search for efficient tools for the environmentally benign detection of OPs (Li et al., 2014; de Castro et al., 2016). The mechanism of action of organophosphates (OPs), as well as their metabolites, has been studied using experimental and computational methods. They indicated that hydrogen bonds between P=O and oxyanion hole was critical for AChE inhibition through covalent bond (Zhang et al., 2002; Lee and Barron, 2016). The oxyanion hole (*i.e.* Gly120, Gly121 and Gly122) contributed to the functional architecture and to the hydrolytic efficiency of human AChE (Ordentlich et al., 1998). Compared to P=S

forms, P=O compounds are more active. The reason is that oxidation of P=S to the corresponding P=O results in a more electronegative phosphorus atom.

X-ray crystal structure of acetyl cholinesterase (AChE) has been solved together with several structurally diverse co-crystal structures, including tacrine (Harel et al., 1993), 1-benzyl-4-[(5,6-dimethoxy-1-indanon-2-yl)methyl]piperidine (Kryger et al., 1998) and several OPs (Millard et al., 1999a, 1999b). It was a major way to acquire crucial information on the binding modes in the active site of the enzyme. The active site of AChE comprised a catalytic subsite and an anionic subsite, which binds the quaternary group of acetylcholine (Quinn, 1987). Like other serine hydrolases, the catalytic triad is located at the bottom of a deep and narrow cavity, named the "aromatic gorge". The primary site of interaction of the quaternary group of acetylcholine is with the aromatic ring of the Trp84. The x-ray crystallographic evidence showed the presence of Trp84 and Phe330 in the "anionic" subsite of the active site and a distal residue, Trp279, near the top of the gorge, was also a part of the "peripheral" anionic site (Sussman et al., 1991). Molecular simulation approach was another way to investigate the orientation of these inhibitors to AChE (Rosenberry et al., 1999; Pilger et al., 2001; Dvir et al., 2002; Cavalli et al., 2004). This process occurred in an enantioselective manner, as exemplified by the *S*-enantiomer of an

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extremely poisonous gas called sarin, which phosphorylated the tyrosineosine within the active site of AChE at least 4200-fold faster than the corresponding *R*-enantiomer (Boter and Dijk, 1969). A more complicated array of interactions is involved in the phosphorylation of this enzyme to diastereoisomers of soman (Ordentlich et al., 1999). It demonstrates that AChE can discriminate between different enantiomers of OPs in terms of their toxicity towards the active site of AChE.

Enantiospecific toxicity is considered as a part of the risk assessment and regulatory decision-making processes underlying the development and application of chiral pesticides. Several quantitative structure–activity relationship (QSAR) studies have been reported pertaining to the specificity and orientation of tetrahedral alkyl phosphate in cholinesterase (Millard et al., 1999a; Ordentlich et al., 2005). Authors of these studies tried to explain the relationship between the shape of the active site of the AChE enzyme and the stereo selectivity of its binding interactions. These events were dependent on the dimensions of the acyl pocket, which governed the leaving group orientation and the productive association of the phosphonyl oxygen with the oxyanion hole (Wong et al., 2000). Comparative molecular field analysis has also been used to model the binding affinities of 30 OPs, but the researchers neglected to consider the differences in the stereo chemistries of the OPs when overlaying the different conformations (Guo et al., 2006). With this in mind, the aim of the current study was to develop a new molecular modeling method for the discovery of new AChE inhibitors. Subsequent determination of the inhibitory activities of these compounds against AChE by use of an *in vitro* bioassay would be used to confirm the validity of this approach. Different enantiomers of chiral OPs can be used to probe the enantioselectivity of the binding pocket of AChE towards these compounds. The molecular simulation approach developed in this study could therefore be used to predict the enantiospecificity of AChE towards various chiral compounds, which can be otherwise difficult to determine experimentally.

2. Materials and methods

2.1. Reagents

Ethyl *p*-nitrophenyl phenylphosphonate (EPN, 96.5%) was purchased from the Dr Ehrenstorfer Co. (Augsburg, Germany). Human AChE (EC 3.1.1.7) expressed in human embryonic kidney 293, acetylthiocholine iodide ($\geq 98\%$), 1-naphthaleneacetic anhydride (96%), *N*,4-dimethyl-*N*-phenyl-benzenesulfonamide ($\geq 96\%$), tacrine hydrochloride ($\geq 99\%$), dimethyl sulfoxide (DMSO, $\geq 99\%$), 2,2'-dinitro-5,5'-dithiobenzoic acid (DTNB, $\geq 97.5\%$) and sodium dodecyl sulfate ($\geq 98.5\%$) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate buffer solution (pH 8.0) was prepared by use of solutions of NaCl, KCl, KH_2PO_4 and Na_2HPO_4 . All of the reagents used in this study were prepared as the analytical grade.

2.2. Theoretical calculations

Six pairs of chiral organophosphates evaluated in this study (Hosea

et al., 1995; Yen et al., 2003; Liu et al., 2006; Nillos et al., 2007) were shown in Table 1, where the *S/R*-activities had been described as the quotient of the *S*- and *R*-activities. Three dimensional 3D structures of these OPs were constructed by use of the SYBYL X1.1 molecular modeling software package (Tripos, Princeton, NJ 08540, USA). Chemical structures were first geometrically optimized by use of the standard Tripos force field with a conjugate-gradient energy convergence criterion of 0.05 kcal/mol. Atomic charges were calculated by use of the Gasteiger + Hückel charge field. The X-ray crystal structure of human AChE was obtained from the Protein Data Bank (<http://www.rcsb.org/pdb>), in which the protein data file entitled accession number 2×8B (Carletti et al., 2010). Biopolymer package was used in the current study to identify the key amino acid residues involved in the binding of the co-crystallized ligand to the protein. Amino acid residues within 6.5 Å around the ligand were considered during this process. Different binding modes of the chiral OPs to AChE were investigated by use of the Surflex-Dock program (Jain, 2003). Surflex-Dock's scoring function, which was based on hydrophobic, polar, repulsive, entropic and salvation terms, was trained as the total score (TS). Total score was then used to estimate the dissociation constant K_d , which was expressed in units of $-\log K_d$. In addition to the automated docking procedure, this function was recently enhanced by the incorporation of a base fragment matching algorithm. In this case, the fragments are allowed to shift from their original position during the pose optimization process.

A DISCOtech program was used to align bioactive conformations, including those of *R*-trichloronate, *S*-leptophos, *R*-fonofos, *S*-cycloheptyl thiocholine, *S*-isopropyl thiocholine, and *S*-cycloheptyl methyl *S*-ethyl phosphonyl thioate. Bioactive conformations of these compounds generated from the Surflex-Dock program were treated as the initial configurations for establishing 50 low energy conformations. The DISCOtech program initially assigned as a series of pharmacophore features, such as hydrogen bond donor atoms, hydrogen bond acceptor atoms, charged centers, mass centers of the hydrophobic rings, and the most likely locations of the binding sites. In some cases, the chirality check option was switched on prior to performing the search process, although this was dependent on the multi-search method. In this study, the most common substructure for elements within a series of aligned conformations was superimposed by use of the categories algorithm. The 'features by class' option was defined and the number of pharmacophore features required in each class was set to more than two donor sites, as well as one acceptor atom, and one hydrophobic center. Distance tolerance between two pharmacophore features in the overlaid structures was set to 0.25 Å, which was increased up to 0.5 Å if no model was found by use of the lower tolerance settings (step size 0.25 Å). The National Cancer Institute database was searched by use of the UNITY program, which contained two hundred thousand chemical structures at the time of this search.

2.3. AChE competitive inhibition assay

A colorimetric assay based on the DTNB-mediated reduction of acetylthiocholine iodide was used to measure the inhibitory activity of

Table 1

Observed vs. predicted *S/R*-enantiomers of organophosphates docked with acetyl cholinesterase (AChE).

OPs	<i>S</i> -activity	<i>R</i> -activity	Observed <i>S/R</i>	<i>S</i> -docking	<i>R</i> -docking	Predicted <i>S/R</i>
trichloronate	8.40 ± 0.12 ^a	9.31 ± 0.13 ^a	0.90	4.82	5.37	0.90
leptophos	10.05 ^a	8.74 ^a	1.15	5.46	4.88	1.12
fonofos	7.85 ± 0.01 ^a	9.04 ± 0.10 ^a	0.87	4.13	4.62	0.89
cycloheptyl thiocholine	8.28 ± 0.07 ^b	5.91 ± 0.05 ^b	1.40	7.65	5.78	1.32
isopropyl thiocholine	7.20 ± 0.03 ^b	5.15 ± 0.10 ^b	1.40	3.99	2.92	1.37
cycloheptyl methyl <i>S</i> -ethyl phosphonyl thioate	4.88 ± 0.03 ^b	2.25 ± 0.05 ^b	2.17	5.40	3.23	1.67

Note:

^a The logarithm of the concentration that caused 50% mortality of the test population (μg/L) (Yen et al., 2003; Liu et al., 2006; Nillos et al., 2007);

^b The logarithm of bimolecular rate constants ($\text{min}^{-1} \text{M}^{-1}$) determined for the inhibition of cholinesterase by *S*- and *R*-enantiomers (Hosea et al., 1995).

these compounds against AChE (Ellman et al., 1961). The reduction of acetylthiocholine iodide with DTNB resulted in the formation of thiocholine, which gave a yellow anion as an indicator of enzymatic activity. All of these experiments were conducted in triplicate, with each experiment including a positive control (tacrine) and a solvent blank control (DMSO). Forty micro liters of AChE (0.01 U/ μ L) in phosphate buffer and 100 micro liters of DTNB (2.5 mmol/L) were added to the wells of a 96-well micro plate. Twenty micro liters of tacrine in DMSO was added to each well, followed a solution of the sample extracts in DMSO (1.10 g/mL, $\geq 99.9\%$), and the plate was then cultured in a thermo shaker (PST-60 HL plus, BIOSAN, Warren, MI 48091-1351, Latvia) at 310 K for 0.5 h. Serineal dilutions of tacrine (in DMSO) were conducted in each series of experiments for concentrations in the range of 10^{-8} to 10 mM to obtain a standard calibration curve. Thirty micro liters of acetylcholine iodide (7.5 mM) was added to each well to initiate the reduction reaction and the development of the yellow color associated with the formation of the anionic species was recorded over time. The reaction was terminated by adding 10 μ L of a 4% (w/v) solution of sodium dodecyl sulfate in H₂O.

Optical density (OD) of the supernatant was measured by use of a micro plate reader (Biotech, Winooski, VT 05404, USA) at 412 nm. The AChE inhibitory activity was defined as follows:

$$U = \frac{A_B - A_S}{A_B} \times 100\% \quad (1)$$

where U is the percentage of AChE inhibitory activity, A_B is the OD of the supernatant from the enzymatic reaction of the blank at 412 nm, and A_S is the OD of the supernatant from the enzymatic reaction of the sample at 412 nm.

2.4. Measurement of enantiospecific impact on the active site of AChE

Fluorescent properties of AChE were measured by use of an F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). One hundred micro liters of AChE was added to 900 micro liters of sodium phosphate buffer (0.1 mol/L), and the resulting mixture was incubated on a thermo shaker at 37 °C for 30 min. DMSO solutions of the samples (1 mmol/L) were added to this mixture, and 3D scan procedures for fluorescent intensity were monitored with an excitation wavelengths of 200–320 nm and emission wavelengths of 250–400 nm. Scan speed were set at 30,000 nm/min and PMT voltage was 550 V. Slit widths for both wavelengths were set at 5 nm. Assays were performed at 30 s intervals throughout the 3D scan process with 30 replicates.

The Stern–Volmer equation was used to investigate the fluorescence quenching in terms of quenching dynamics (Sklar et al., 1977). According to this equation, F and F_0 represented the fluorescent intensities in the absence and presence of quenchable reagent, respectively. K_q was a quenching speed constant and C_q was the concentration of quenchable reagent. The parameter τ_0 was calculated as the average fluorescence lifetime, but it was usually set at 10^{-8} s for biological macromolecules.

$$F_0/F = 1 + K_q \tau_0 C_q \quad (2)$$

Binding distances between fluorescent chemicals and quenchable reagents were also calculated by use of the Förster energy transfer equation, as follows:

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r_0^6) \quad (3)$$

where E represented the energy transfer efficiency, R_0 was the critical distance when E was equal to 50%, and r_0 is the actual binding distance.

2.5. Statistical analysis

Dose-response curve was fitted by use of a Sigmoidal Boltzmann model (see equation below) to obtain the effective concentration (EC)

values (Rehmann et al., 1999),

$$EC = A_2 + \frac{A_1 - A_2}{1 + \exp\left(\frac{x - x_0}{d_x}\right)} \quad (4)$$

where A_1 was the initial value, A_2 was the final value, x_0 was the center value, and d_x was the time constant (Table S1).

3. Results and discussion

3.1. Binding mode of chiral OPs to human AChE

Six pairs of OP enantiomers were successfully docked into the active site of human AChE by use of the Surflex-Dock programs. As shown in Table 1, the binding affinities of all these compounds were predicted. The S/R -activities of the six OPs revealed that they all exhibited enantiospecific inhibition towards human AChE. The docking scores revealed that *R*-trichloronate, *S*-leptophos, *R*-fonofos, *S*-cycloheptyl methyl *S*-ethyl phosphonyl, *S*-isopropyl thiocholine and *S*-cycloheptyl thiocholine had higher binding affinities than the corresponding enantiomers. The simulated complexes suggested that Tryptophan86, Glycine120, Glycine121, Serine125, Glutamic acid202, Serine203, Tyrosine337 and Histidine447 were the key amino acid residues to form meaningful interactions with the 12 OPs (Fig. 1). Serine125 and Tyrosine337 were key H-bonding sites for the stabilization of ligands embedded in the binding site of AChE. Volume of active pocket was also considered in terms of the lipophilic potential surface of the active pocket, in which six pairs of enantiomers possessed different postures in the active site. Conformations of the bioactive conformers of different OPs revealed that their chiral center was closer to the catalytic triad (i.e., Glutamic acid202, Serine203, and Histidine447) than it was in the corresponding inactive conformations. The modeling results also revealed that the formation of an H-bonding interaction between OPs and Serine125 or Tyrosine337 enhanced the binding affinity of these compounds to human AChE. A comparison of the binding interactions of trichloronate (Fig. 1a) with those of leptophos (Fig. 1b) revealed that they both formed H-bond interactions to Serine125, but the hydroxyl group of the two bioactive enantiomers were closer to the oxyanion hole formed by Glycine120, Glycine121 and Serine125 than that of inactive enantiomers. It is noteworthy that a benzene ring bearing chlorine substituent would not be able to fit into the bottom of the active site due to the steric exclusion effect. There were common features among six bioactive conformations. The benzene ring in fonofos and the alicyclic ring in *S*-ethyl were parallel to the plane of the hydrophobic tryptophan86 residue, suggesting that *R*-fonofos and *S*-cycloheptyl methyl *S*-ethyl phosphonyl were the major conformations (Fig. 1c and d). The bioactive conformations shown in Fig. 1e and f revealed that the chiral phosphorus atoms of these OPs were located in a narrow valley and that the chlorine leaving group pointed away from the entrance to the binding site. It is noteworthy that the AChE enzyme achieves enantiospecific recognition through a combination of specific hydrogen bonding interactions and complementary hydrophobic interactions with the ligand. Different binding modes of the two enantiomers could therefore be used to predict the inhibitory activity of new OPs towards AChE. The calculated non-bonding binding energies of these compounds could be used to explain the structure activity relationships of chiral OPs to interact with human AChE.

3.2. A novel enantiomer-based virtual screening to discover AChE inhibitors

A credible pharmacophore model was then established to investigate the toxic mechanisms of chiral OPs responsible for the enantioselectivity exhibited by AChE. The chemical feature-based pharmacophore model based on six bioactive enantiomers contained two hydrogen bond donors, a hydrogen bond acceptor and a hydrophobic feature

Fig. 1. Conformations of organophosphates (OPs) enantiomers docked into human AChE, including trichloronate (a), leptophos (b), fonofos (c), cycloheptyl thiocholine (d), isopropyl thiocholine (e), and cycloheptyl methyl S-ethyl phosphonyl thioate (f). The structure of AChE is shown as a ribbon diagram and the compound in a stick representation. Conformation of OPs with atom-type color is bioactive enantiomer but another enantiomer is depicted with red color. Yellow dashed lines indicate the hydrogen bonds and lipophilic potential (LP) surfaces of active pocket are characterized with color ramps. The key amino acid residues are colored cyan.

(Fig. 2). The total score of this model was 0.31 with a tolerance of 1.25 Å. Meanwhile, other six non-bioactive enantiomers for pharmacophore were not within a tolerance of 1.25 Å, which was demonstrated as a meaningful virtual screening. A four-feature pharmacophore model developed in a previous study composed of hydrophobic regions and H-bond donor/acceptor and provided valuable insight for designing selective inhibitors for the treatment of Alzheimer's disease (Valasani et al., 2013). Barron et al. developed a structure-based pharmacophore model and generated 3D-fingerprint to describe 3D protein-ligand interactions in the active site gorge of AChE. The eight pharmacophore features in the binding site of AChE included 3 aromatic/hydrogen bond donor or hydrophobic features that interacted with Trp84 and Phe330, a hydrogen bond donor feature that interacted with carbonyl oxygen of His440, a hydrogen bond acceptor feature that interacted with Gly118, Gly119, and Ala201, and 3 hydrophobic features in acyl pocket (Phe288 and Phe290) (Lee and Barron, 2015). The type of those pharmacophore models also contained four location-specific features

with stereospecificity (Mesecar and Koshland, 2000). It was concluded that the tetrahedral structure was a basis to determine the enantiospecificity of target enzyme. It seemed plausible that the primary 3D pharmacophore model could provide a good platform for further database searching and the rational design of new AChE inhibitors.

The four-location model developed in the current study was used to conduct a searching query against the National Cancer Institute (NCI) database for anti-HIV and anti-cancer assay data. One of the main advantages of virtual screening is that it allows for the rapid and low-cost screening of large numbers of compounds *in silico*, making it a useful tool for the prescreening of potential lead compounds. Tolerance value of the pharmacophore model was modified from 0.75 to 0.25 Å in this particular case to enhance its accuracy. Twenty-four compounds were found, including 1-naphthylacetic anhydride (M10202), 2-(di-methylamino)-2-oxo-1-phenylethyl(phenyl)phosphinic acid (M1300-76), ethyl bis(2,2-dimethyl-1-aziridinyl)phosphorylcarbamate (M5132-5), dimethyl bicyclo[2.2.1]hept-2-ylphosphonate (M 138990), methyl

Fig. 2. A pharmacophore model for six bioactive enantiomers of OPs. It contains two hydrogen-bond donors, a hydrogen-bond acceptor and a hydrophobic feature. Yellow lines depict the maximum common substructures.

(diphenylphosphorothioyl)(phenyl)acetate (M130079), O-ethyl O-(4-(hydroxy(oxido)amino)phenyl) phenylphosphonothioate (M404840), 4-methylbenzenesulfonic acid (4871), ethyl 2-((phenylsulfonyl)methyl)acrylate (M623994), 1-tert-butyl-4-((chlorooxy)sulfinyl)benzene (M8544), No name (M122496), N-(cyclobutyl(1-pyrrolidinyl)methylene)-8-quinolinesulfonamide (M116974), N'-benzoyl-N'-methylbenzenesulfonohydrazide (M37428), 1-(((bis(2-chloroethyl)amino)oxy)sulfinyl)-4-methylbenzene (M23809), 7-((phenylsulfonyl)amino)bicyclo[2.2.1]hept-2-yl acetate (M98587), N-(4-aminophenyl)-N,4-dimethylbenzenesulfonamide (M105638), N,4-dimethyl-N-phenylbenzenesulfonamide (M2225), 4-aminophenylphosphonous acid (M3625), 2-(1,2-dihydro-5-acenaphthylenyl)acetamide (M17466), 1-(4-(methylthio)phenyl)-2-(1-quinolin-1-yl)ethanone (M35029), (2-hydroxy-3-methoxy-5-methylphenyl)(4-phenyl-4,5-dihydro-1H-pyrazol-3-yl)methanone (M404854), 2-(1,2,2-triisopropoxyethoxy)propane (M21542), allyl 3-phenyl-2-oxiranecarboxylate (M68483), diethyl 2,2-dibenzylmalonate (M8726), and dimethyl 8-oxo-2-cyclooctene-1,2-dicarboxylate (M119539). Most of those compounds contained chiral or pro-chiral atoms. Three structurally diverse compounds from this small set were purchased to measure their inhibitory activity towards AChE. We would have preferred to purchase all of these compounds to measure their inhibitory activity towards AChE, but some of these compounds were not commercially available.

3.3. Measurement of AChE activity by competitive inhibitory assay and fluorescence assay

Potency of OPs to inhibit activity of AChE was measured and compared to theoretical results. Toxicities of these three compounds were evaluated by use of an AChE competitive inhibitory assay, which allowed us to generate dose response curves of them. They were EPN, 1-naphthaleneacetic anhydride and N, 4-dimethyl-N-phenyl-benzenesulfonamide. Tacrine hydrochloride was also tested as positive control. This method has been used routinely for many years to monitor terrestrial and freshwater aquatic systems as an indicator for the bioactivity of OPs (Fulton and Key, 2001). As shown in Fig. 3, the logarithm of median effective concentration of tacrine (positive control) was determined as -6.48 . 1-naphthaleneacetic anhydride, EPN and

N, 4-dimethyl-N-phenyl-benzenesulfonamide exhibited moderate inhibitory activity towards AChE compared with tacrine, with $\log EC_{50}$ values of -2.91 , -2.46 and -5.22 , respectively. The activity of 1-naphthaleneacetic anhydride was attributed to its hydrophobic interaction with AChE, whereas the pro-chiral N, 4-dimethyl-N-phenyl-benzenesulfonamide compound has higher activities. Meanwhile, our measurement for EPN is consistent with reports from the literature concerning the toxicity of this compound towards mammals (Devillers, 2004). However, the different enantiomers of this compound have been reported to show different toxicities (Johnson and Read, 1987; Tsugawa et al., 2000). Further study is therefore required to assess the environmental risks associated with the different enantiomers of EPN.

A fluorescence assay on human AChE was performed by use of an F-7000 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). As shown in Fig. 4a, fluorescence spectrum of AChE contained an emission peak at 320 nm with an excitation wavelength of 230 nm, which is a typical characteristic of the tryptophan. However, the result of a 3D fluorescence lifetime scan in the presence of EPN with a fixed excitation wavelength of 270 nm and a reaction time of 900 s revealed that the fluorescence intensity of tryptophan decreased from 333.8 nm to 128.6 nm (Fig. 4b). This finding indicated that fluorescence had been quenched during the reaction time. The K_q value of this system was determined to be 1.61×10^{12} L/mol s in Eq. (2), which was much greater than the maximum value allowed for a macromolecule of 2.0×10^{10} L/mol s. Fluorescence quenching of AChE was therefore attributed to the formation of an EPN-AChE complex, that was involved in static quenching. The binding distance r_0 was determined to be $0.924 R_0$ ($0.5 R_0 < r_0 < 1.5 R_0$), which gave an energy-transfer efficiency E of 61.6% in Eq. (3). It suggested that EPN was an effective quenchable reagent for AChE, because it might bind to its hydrophobic cavity. Compared with the fluorescence quenching of Tyrosine, fluorescence quenching of tryptophan was greater, with a considerable Einstein shift occurring during the quenching process. This phenomenon shows that the tryptophan plays an important role in the binding of EPN to AChE, and that the interaction between these two groups is weaker than that expected based on the Einstein shift.

Given that an isomer of EPN could lead to a greater decrease in the polarity of tryptophan, it should be possible to confirm the differences

Fig. 3. Dose-response curves for AChE inhibitory activity. Tacrine hydrochloride is the positive control and others are tested compounds. Each concentration was repeated three times. Error bars indicate standard error. Modeled data for the compounds are shown as a solid line.

in the binding of enantiomers without chiral separation. Binding modes of *S*- and *R*-EPN were predicted by use of a flexible docking method. As shown in Fig. 5, *S*-EPN was buried deep inside the hydrophobic cavity of human AChE, with a water molecule acting as a hydrogen bond bridge between *S*-EPN and Serine125. The benzene ring of *S*-EPN was located in close proximity to the hydrophobic environment of Tryptophan86 and it could form credible π - π interactions with the bottom of the pocket. In contrast, *R*-EPN adopted the opposite conformation to *S*-EPN, with its nitrobenzene substituent occupying the catalytic site, making it difficult to form a catalytic intermediate through a covalent process. These findings therefore confirmed that *S*-

EPN exhibited greater inhibitory activity towards AChE than *R*-EPN.

4. Conclusions

Molecular simulation, on basis of stereochemistry of enzyme, provides a sensitive process to identify the bioactive enantiomers of OPs to AChE. A virtual screening method was then developed to search new inhibitors to human AChE, including EPN, 1-naphthaleneacetic anhydride and *N*, 4-dimethyl-*N*-phenyl-benzenesulfonamide. Some *in vitro* assays were performed to determine the inhibitory activity as well as the binding modes of different enantiomers. This study gives a novel

Fig. 4. 3-D fluorescence scan contour (a) and 3-D time scan contour (b) for fluorescence quenching of AChE in the presence of ethyl *p*-nitrophenyl phenylphosphonate (EPN).

Fig. 5. Different binding modes between S-EPN and R-EPN, which are described by use of LIGPLOT (Wallace et al., 1995). The key mechanism of action in present study includes hydrogen bond and its length, hydrophobic contacts and solvent accessibility.

direction to evaluate the stereo selective toxicity of chiral compounds to their target proteins.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2016.12.035.

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